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The NF2 tumor suppressor protein, termed Merlin/Schwanomin, is a member of the protein 4.1 superfamily that functions as a linker of the plasma membrane to the cytoskeleton. A major limitation in understanding the biochemical function of NF2 protein is the unavailability of purified native protein from human tissues. Our discovery of the presence of NF2 protein in human erythrocytes allowed us to develop a purification procedure that affords homogeneous NF2 protein in its native state. Selective extraction of ghosts in the low ionic strength buffer indicated that the bulk of NF2 protein remains associated with spectrin-actin depleted inside-out-vesicles. Quantitative removal of NF2 protein by potassium iodide shows that it is tightly associated with the membrane. Interestingly, the NF2 protein was completely solubilized in 0.5% Triton X-100 at high but not at low ionic strength. These results suggest a novel mode of NF2 protein association with the membrane that is distinct from protein 4.1. We devised a purification strategy to isolate NF2 protein from human erythrocytes. Identification of NF2 protein as a constituent of erythrocyte membrane provides a unique opportunity to study the biochemical properties of native protein, and identify proteins that interact with NF2 protein in the mammalian plasma membrane.

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Progress Report

Career Development Award # NF020087
PI: Athar H. Chishti
Title: Biochemical characterization of native Schwannomin/Merlin

OBJECTIVE

As outlined in the Statement of Work (SOW), Task #1 proposed purification of native NF2 protein from human erythrocyte membranes. We have accomplished this task, and current experiments are investigating the biochemical properties of purified NF2 protein.

SUMMARY OF THE WORK COMPLETED

Neurofibromatosis type 2 (NF2) is an autosomal dominantly inherited disorder predisposing individuals to develop tumors of neuronal origin. The NF2 tumor suppressor protein, termed Merlin/Schwanomin, is a member of the protein 4.1 superfamily that functions as a linker of the plasma membrane to the cortical cytoskeleton. A major limitation in understanding the biochemical properties of NF2 protein is the unavailability of purified native protein from human tissues. This limitation is further exacerbated by the difficulty in expressing functionally active NF2 protein using recombinant cDNA approaches. Our discovery of the presence of NF2 protein in the erythrocytes allowed us to develop a purification procedure that affords homogenous NF2 protein in its native state. Western blot analysis revealed the presence of a single ~70 kDa polypeptide in the erythrocyte ghosts. Selective extraction of ghosts in the low ionic strength buffer indicated that the bulk of NF2 protein remains associated with spectrin-actin depleted inside-out-vesicles (IOVs). Quantitative removal of NF2 protein by extraction of (IOVs) in 1.0 M potassium iodide shows that it is tightly associated with the membrane. Interestingly, the NF2 protein was completely solubilized from ghosts in 0.5% Triton X-100 at high ionic strength (0.5 M KCl) but not at low ionic strength. These results suggest a novel mode of NF2 protein association with the erythrocyte membrane that is distinct from protein 4.1. Based on these biochemical properties, we have devised a purification strategy that allows isolation of cytoskeleton-associated NF2 protein from human erythrocyte ghosts. Identification of the NF2 protein as a constituent of erythrocyte membrane provides a unique opportunity to study the biochemical properties of native protein, and identify proteins that bind to the NF2 protein in the mammalian plasma membrane.

EXPERIMENTAL PROCEDURES AND RESULTS

Preparation of Ghosts: Human red blood cells were washed four times with 5.0 mM sodium phosphate, pH 8.0; 150 mM NaCl, and 0.1 mM EGTA. The buffy coat was carefully removed manually as well as through the use of mixed cellulose chromatography and erythrocytes were lysed in 20 volumes of 5.0 mM sodium phosphate; pH 8.0, 0.1 mM EGTA; 1 mM PMSF. Lysed cells were pelleted by centrifugation (20 minutes at 20,000 x g) and the membranes (ghosts) were washed several times with the lysis buffer to remove hemoglobin.

SDS-PAGE and Immunoblot Analysis: Proteins were resolved by SDS-PAGE (10% acrylamide) and electro-transferred onto the nitrocellulose membrane. The nitrocellulose blot were blocked with the blocking buffer (6% casein; 1% polyvinyl pyrrolidone-40; 10 mM EDTA in PBS, pH 7.4) and incubated with anti-NF2 antibodies at 1:1000 dilution in the blocking buffer for 2 hours at room temperature. The NF2 antibody was raised by injecting the synthetic peptide of human NF2 protein into rabbits. This antibody is specific for the NF2 protein isoform 1 (SCH1). Following incubation, blots were washed with the TBS-T buffer (10 mM Tris-HCl, pH 7.5; 150 mM NaCl and 0.1% Tween-20), and incubated for 1 hour with goat anti-rabbit IgG-horseradish peroxidase at 1: 1500 in blocking buffer. After washing with the TBS-T, the NF2 protein was visualized using the ECL Western blotting detection system.

Triton X-100 mediated solubilization of human erythrocyte ghosts: All samples were analyzed in duplicate. Solubilization buffer (SB) consists of 50 mM Tris-HCl, pH 7.5, 1.0 mM EDTA, 1.0 mM EGTA, protease inhibitors. It is noteworthy that the NF2 protein remains associated with the cytoskeleton after extraction with Triton X-100. However, extraction of ghosts with 0.5% Triton X-100 containing 500 mM KCl completely solubilized the NF2 protein.

Extraction of cytoskeleton-associated NF2 protein by detergent- free salt solution: Ghosts were extracted with 0.5% Triton X-100 containing 150 mM KCl. Cytoskeletons were recovered by centrifugation on a cushion of sucrose. Detergent-free cytoskeletons were then extracted with an increasing concentration of KCl. The rationale of this experiment was to test whether the NF2 protein could be extracted by high salt without the use of detergent. If so, then does it co-extract with erythrocyte proteins such as ankyrin and adducin. The NF2 protein was not extracted from the detergent-extracted cytoskeleton by simple salt solution. In contrast, a significant amount of ankyrin was eluted under similar extraction conditions.

Purification of NF2 protein from the Triton extracted cytoskeleton: Triton extracted cytoskeleton was disrupted by incubation in 10 volumes of low ionic strength buffer (3 mM Tris-HCl, pH 8.5, 0.5 mM EDTA, 2 mM DTT) for 45 minutes at 37°C followed by centrifugation at 40,000 rpm for 30 minutes. The supernatant containing cytoskeletal proteins was chromatographed on DEAE-Sephacel column (2.5 x 50 cm) that had been previously equilibrated with the column buffer (20 mM Tris-HCl, pH 8.3, 1 mM EGTA, 1 mM DTT and 20 mM KCl). The bound proteins were eluted with 800 ml linear gradient of 20-500 mM KCl in column buffer. The fractions were analyzed by SDS-PAGE, followed by Coomassie blue staining and western blot analysis. The fractions rich in NF2 protein were pooled and dialyzed, and further chromatographed on a Mono Q column. The column was developed with a 50 ml linear gradient of 20-400 mM KCl. The fractions rich in NF2 protein were pooled, dialyzed, and concentrated using a membrane concentrator. The concentrated preparation was analyzed by SDS-PAGE. Purity of NF2 protein was checked by gel electrophoresis and immunoblotting. Note that the purified preparation shows only a single band corresponding to NF2 protein.

Triton X-100 extracts NF2 protein from ghosts only in the presence of 0.5 M potassium chloride.

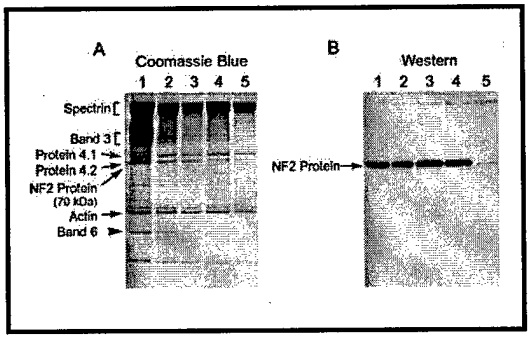

Figure 1: Solubilization buffer consists of 50 mM Tris-HCl, pH 7.5, 1.0 mM EDTA, 1.0 mM EGTA, protease inhibitors. Lane 1 (ghosts); 2 (ghosts extracted with 0.5% Triton in SB); 3 (ghosts extracted with 0.5% Triton + 50 mM KCl in SB); 4 (ghosts extracted with 0.5% Triton + 150 mM KCl in SB); 5 (ghosts extracted with 0.5% Triton + 500 mM KCl in SB).

Cytoskeleton-associated NF2 protein is not extracted by simple salt solution.

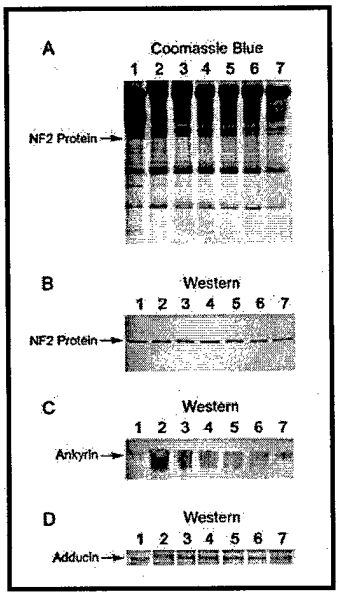

Figure 2: Ghosts were extracted with 0.5% Triton X-100 containing 150 mM KCl. Cytoskeletons were recovered by centrifugation on a cushion of sucrose. Detergent-free cytoskeletons were then extracted with an increasing concentration of KCl. Lane 1 (ghosts); 2 (cytoskeletons); 3 (200 mM KCl); 4 (400 mM KCl); 5 (600 mM KCl); 6 (800 mM KCl); 7 (1000 mM KCl). The NF2 protein is not extracted from the detergent-extracted cytoskeleton by simple salt solution. In contrast, a significant amount of ankyrin and adducin was eluted under similar extraction conditions (panels C and D).

NF2 protein from Triton X-100 extracted cytoskeleton is purified to near homogeneity.

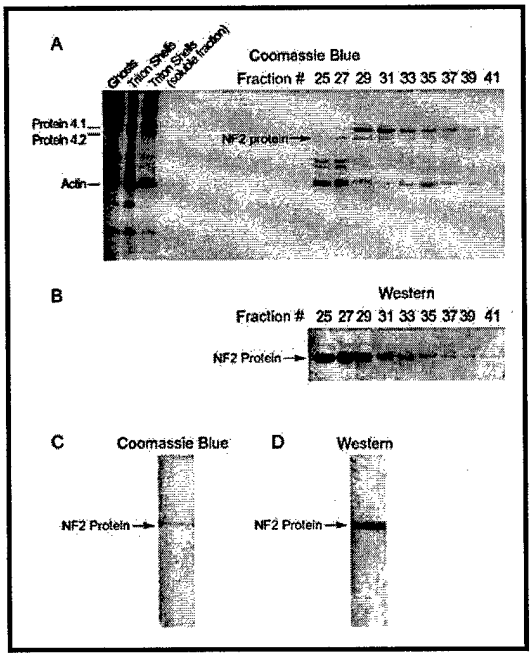

Figure 3: Triton extracted cytoskeleton was disrupted and the supernatant containing cytoskeletal proteins was chromatographed on DEAE-Sephacel column. The bound proteins were eluted with a linear gradient of 20-500 mM KCl in the column buffer. The fractions were analyzed by SDS-PAGE and Western blot analysis. The fractions rich in NF2 protein were pooled, dialyzed, and chromatographed on a Mono Q column. The concentrated protein was analyzed by gel electrophoresis (C) and Western blotting (D).

CONCLUSIONS

- NF2 protein migrates as a single polypeptide of ~ 70 kDa as detected by SDS-PAGE and Western blotting.
- Complete solubilization of the NF2 protein from cytoskeletons with 0.5% Triton X-100 in the presence of 500 mM KCl but not in the absence of Triton X-100 suggests that the NF2 protein may directly interact with membrane lipids in the erythrocyte plasma membrane.
- Release of the bulk of NF2 protein by extraction of IOVs in 1.0 M potassium iodide suggests that the NF2 protein is a peripheral protein that is tightly associated with the plasma membrane.
- Based on the biochemical properties of the NF2 protein, two purification strategies are designed. Approximately, 30,000 copies of NF2 protein are present in each erythrocyte.
- The presence of NF2 protein in the membranes of mutant red cells suggests that the major skeletal membrane proteins such as protein 4.1, glycophorin C, p55, ankyrin, alpha spectrin, band 3, and protein 4.2 are not required for the binding of NF2 protein to the plasma membrane. This result implies that novel binding partner(s) for NF2 protein may exist in the human erythrocytes.